ehp ENVIRONMENTAL HEALTH PERSPECTIVES
http://www.ehponline.org

Early Postnatal Exposure to Ultrafine Particulate Matter Air Pollution: Persistent Ventriculomegaly, Neurochemical Disruption, and Glial Activation Preferentially in Male Mice

Joshua L. Allen, Xiufang Liu, Sean Pelkowski, Brian Palmer, Katherine Conrad, Günter Oberdörster, Douglas Weston, Margot Mayer-Pröschel, and Deborah A. Cory-Slechta

http://dx.doi.org/10.1289/ehp.1307984
Received: 10 December 2013
Accepted: 22 May 2014
Advance Publication: 5 June 2014

NIH National Institute of Environmental Health Sciences

Early Postnatal Exposure to Ultrafine Particulate Matter Air Pollution: Persistent Ventriculomegaly, Neurochemical Disruption, and Glial Activation Preferentially in Male Mice

Joshua L. Allen,[1] Xiufang Liu,[1] Sean Pelkowski,[1] Brian Palmer,[1] Katherine Conrad,[1] Günter Oberdörster,[1] Douglas Weston,[1] Margot Mayer-Pröschel,[2] and Deborah A. Cory-Slechta[1]

[1]Departments of Environmental Medicine and [2]Biomedical Genetics, University of Rochester School of Medicine, Rochester, New York, USA

Address correspondence to Deborah A Cory-Slechta, 601 Elmwood Ave., Box EHSC/Room 2-6810, Rochester, NY 14642. Telephone: 585-275-7060. Fax: 585-246-2591. E-mail: deborah_cory-slechta@urmc.rochester.edu

Running Title: Air Pollution, Ventriculomegaly, and Neurochemical Disruption

Acknowledgments: This work was supported by ES012105 (D.A. Cory-Slechta), ES019852 (D.A. Cory-Slechta), ES001247 (T. Gasiewicz), and T32 ES007026 (B.P. Lawrence).

Competing financial interests: No competing financial interests to declare.

Abstract

Background: Air pollution has been associated with adverse neurological and behavioral health effects in children and adults. Recent studies link air pollutant exposure to adverse neurodevelopmental outcomes, including increased risk for autism, cognitive decline, ischemic stroke, schizophrenia, and depression.

Objectives*:* This study sought to investigate the mechanism(s) by which exposure to concentrated ambient ultrafine particles (CAPS) adversely influence central nervous system (CNS) development.

Methods: C57Bl6/J mice were exposed to ultrafine (<100 nm) CAPS using the Harvard University Concentrated Ambient Particle System or filtered air postnatal days (PND) 4-7 and 10-13 after which animals were euthanized either 24 hours or 40 days following cessation of exposure, and in another group of males at 270 days (ventricle area). Lateral ventricle area, glial activation, CNS cytokines, and monoamine and amino acid neurotransmitters were quantified.

Results: CAPS induced ventriculomegaly (i.e., lateral ventricle dilation) preferentially in male mice that persisted through young adulthood. Additionally, CAPS-exposed males generally showed decreases in developmentally important CNS cytokines, whereas, in females, CAPS induced a neuroinflammatory response as indicated by increases in CNS cytokines. CAPS also induced changes in CNS neurotransmitters and glial activation across multiple brain regions in a sex-dependent manner and increases hippocampal glutamate in males.

Conclusions: CAPS induces brain region- and sex-dependent alterations in cytokines and neurotransmitters in both males and females. Lateral ventricle dilation (i.e., ventriculomegaly) is only observed in CAPS-exposed male mice. Ventriculomegaly is a neuropathology that has been associated with poor neurodevelopmental outcome, autism, and schizophrenia. Our findings

suggest alteration of developmentally important neurochemicals and lateral ventricle dilation may be mechanistically related to observations linking ambient air pollutant exposure and adverse neurological/neurodevelopmental outcome in humans.

Introduction

Air pollution has been associated with adverse neurological and behavioral health effects in children and adults. Recent epidemiological studies report associations between exposure to air pollutants and increased risk for autism (Becerra et al. 2013; Volk et al. 2011; Volk et al. 2013), cognitive decline (Power et al. 2011; Weuve et al. 2012), ischemic stroke (Lisabeth et al. 2008; Wellenius et al. 2012), schizophrenia (Pedersen et al. 2004), and depression (Lim et al. 2012). Exposures, in particular to ultrafine ambient particulate matter (UFP; <100 nm diameter), identified as potentially the most toxic constituent of air pollution (Oberdorster 2000), are pervasive and ubiquitous. Increases in neuroinflammation, oxidative stress, and glial activation have been identified as putative mechanisms by which air pollution exposures may impair the central nervous system (CNS) in adults (Block and Calderon-Garciduenas 2009), but such exposures in the context of early brain development, a time frame considered crucial to causation of autism, schizophrenia, and cognitive development remain largely unexplored. Given the potential public health importance of the reported epidemiological associations, it is imperative that the biological plausibility of such early developmental exposures to produce CNS dysfunction and disease be examined. Thus, we hypothesized that exposure of mice to UFP during early postnatal development, a period of rapid brain growth and differentiation, should adversely influence CNS development by mechanisms identified as subserving air pollutant effects.

Methods

Animals, reagents, and exposures

Eight week old male and female C57Bl6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and allowed to acclimate in the housing room for 1 week prior to breeding. Monogamous pairs of mice were bred for 3 days, males were removed and dams remained singly housed with litters until weaning. Weanling mouse pups were exposed to concentrated ambient ultrafine particles (CAPS; <100 nm) using the Harvard University Concentrated Ambient Particle (HUCAPS) described in full elsewhere (Allen et al. 2013). Briefly, animals were exposed to ambient ultrafine particulates in real-time from PND 4-7 and 10-13 for 4 hours per day for 4 days per week between hours 0700 – 1200, times corresponding to peak vehicular traffic outside the intake valve of the instrumentation. Particulates were concentration approximately 10 fold over ambient outdoor air. The HUCAPS system is fitted with a size-selective inlet and a high-volume (5,000 L/min) UFP concentrator that concentrates ambient particles. CAPS and filtered-air treated animals receive identical experimental manipulation. Due to presence of particle impactor in the HUCAPS system, animals in CAPS exposed chamber may be held at slightly higher negative pressure compared to filtered air; however, flow of CAPS-enriched or filtered air is maintained constant in both chambers. Room air is filtered by HEPA filtration (99.99% effective) for filtered air exposed animals. Humidity and temperature in exposure chambers was maintained at 35-40% and 77-79ºF. Particulate mass concentration and counts are reported in Figure 1. Particle counts were obtained using a condensation particle counter (model 3022A; TSI, Shoreview, MN) and mass concentration was calculated using idealized particle density (1.5 g/cm^3). Animals were euthanized by rapid decapitation due to known effect of anesthetics on neurochemistry on PND14 and PND55 to assess immediate and

persistent effects of CAPS on the developing and young adult CNS; an additional group of male brains from a separate exposure study obtained at PND270 was examined for ventricle area. Exposure characteristics for the PND270 exposure group were similar to those for PND14 and PND55 groups. Details are reported in other work (Allen et al. 2014a). To preclude litter specific effects, only a single pup per time point per sex per litter was used in the study. All mice used in this study were treated humanely and with regard for alleviation of suffering and approved by the University of Rochester IACUC.

GFAP and IBA-1 immunostaining and image analysis

Brains were extracted and placed into 4% paraformaldehyde for 24 hours and then placed in 30% sucrose until they sank. Brains were sectioned on a freezing microtome (Microm) at 40 μm thickness in cryoprotectant (30% sucrose, 30% ethylene glycol in 0.1M phosphate buffer) and stored at -20°C until immunostaining. Every sixth section was stained for glial fibrillary acid protein (GFAP) and ionized calcium-binding adapter molecule 1 (IBA-1) to assess global activation of astrocytes and microglia, respectively. Briefly, brain sections were washed of cryoprotectant and placed into primary antibody for GFAP (Millepore , Billerica, MA; AB5804; 1:4000 dilution) or IBA-1 (Wako Chemicals USA, Richmond, VA 016-20001, 1:5000) for 24 hours. For GFAP, tissue was then placed into biotinylated secondary antibody (Vector Labs, Burlingame, CA; BA1000; 1:200 dilution) for 1 hour and the stain was visualized using 3-3'-diaminobenzidine (DAB). For IBA-1, tissue was placed into fluorescently-label secondary (Life Technologies, Grand Island, NY, A-11012; 1:400 dilution). Immunolableled tissue was mounted onto Superfrost Plus micro slides (VWR, Radnor, PA, 48311-703) and coverslipped using

Cytoseal 60 (for chromogenic tissue; Fisher Scientific; Pittsburg, PA; 23-244257) or ProLong Gold Antifade Reagent (Life Technologies, Grand Island, NY, P36930).

Three images of each of following brain regions were obtained: corpus callosum, cortex, ventral midbrain, dentate gyrus, hippocampus (CA1/CA2), and striatum. Relative immunoreactivity was determined using Image Pro Plus 7.0 (MediaCybernetics, Rockville, MD). All images underwent contrast enhancement prior to utilization of the count/size method. Briefly, immunoreactive cells on 2-3 sections per brain region were enumerated using count/size feature of Image Pro Plus 7.0 across 3 equally sized fields per brain region modified from Cao et al. (2012). Data is reported as percent of time point- and sex-matched control.

Lateral ventricle and Aqueduct of Sylvius area determination

The area of the lateral ventricles (Approximate Bregma range 1.10 mm-0.38 mm) and Aqueduct of Sylvius (Approximate Bregma range: -3.88 - -4.84 mm) was determined by tracing the outline of the area of interest in at least 4 adjacent sections of slide-mounted brain using Neurolucida (MBF, Villiston, VT). Software enumerated the area of interest area in μm^2. Lateral ventricle bregma for the PND14 brains are approximate given that, to the knowledge of the authors, no atlas at that point in early postnatal brain development exists. To examine persistence of CAPS-induced lateral ventricle dilation male mice, ventricle area was quantified in another group of identically but not concurrently exposed males from brain harvested at approximately PND270. Unlike the mice from which brains were obtained at PND14 and 55, mice from which PND270 tissue were harvested had undergone behavioral testing (reported in Allen et al 2014a).

Neurotransmitters quantification

Briefly, brains were extracted and dissected on an ice-cold plate into the following regions: olfactory bulb, hippocampus, midbrain, striatum, hypothalamus, cerebellum, and cortex. High performance liquid chromotography coupled with an electrochemical detector (for monoamines) or a fluorescent detector (for amino acids) was used to quantify dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), norepinephrine (NE), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), glutamine (GLN), glutamate (GLU), and γ-aminobutyric acid (GABA), expressed as ng/mg protein. Dopamine turnover was calculated as [DOPAC]/[DA]. Method details are published elsewhere (Cory-Slechta et al. 2013; Cory-Slechta et al. 2004; Virgolini et al. 2008).

Cytokines

Interleukin 1-beta (IL-1β), tumor necrosis factor-alpha (TNFα), and interleukin-6 (IL-6) in the striatum, hippocampus, olfactory bulb, midbrain, cortex, and cerebellum were quantified using custom multi-plex plate-based chemiluminescent ELISA (Quansys Biosciences, Logan, UT). Briefly, tissue was quickly sonicated in 0.1M PBS, pH 7.4 containing 1% protease inhibitor cocktail (Sigma, St. Louis, MO; P8340). 25 µL of brain homogenate was loaded per well and were run in duplicate according to manufacturer's instructions. The chemiluminescent signal was visualized using the Q-view Imager and analyzed using Q-View Software (Quansys Biosciences, Logan, UT). Cytokine levels were normalized to total protein content of the same region as determined by the bicinchoninic acid method.

Statistical analyses

Statistical analysis was carried out using JMP10 (Cary, NC). Lateral ventricle dilation is characterized by a statistical interaction of postnatal CAPS x sex (see results) and thus all statistical analyses were separated by sex and used two-way ANOVAs with age of sacrifice and treatment group as the independent factors. Fisher's LSD post hoc analysis was used in the event of age of sacrifice by treatment interaction. All analyses were performed as two-tailed tests and $p<0.05$ is considered statistically significant.

Results

Mice were exposed from postnatal day (PND) 4-7 and 10 -13 for 4 hrs/day between hours 0700-1200, times corresponding to peak vehicular traffic and particle concentration levels near the intake valve. Mean CAPS count across the 8 days of postnatal exposure was approximately 200,000 particles/cm^3, and the mean particle mass concentration was 96 $\mu g/m^3$ (Figure 1). Particles remained ultrafine (<100 nm diameter) for all exposure days (Figure 1 inset).

To address the potential of CAPS to elicit immediate CNS effects and to determine the persistence of such effects, mice were euthanized at two times following cessation of exposure: PND14 (24 hrs post-exposure) and PND55 (young adulthood). Notably, CAPS-exposed male (TX x Sex interaction: $F(1,32)=10.0559$, $p<0.01$; main effect of CAPS treatment for males ($F(1,16)=10.3298$,$p=0.0054$), but not female ($F(1,16)=0.2455$,$p=0.6270$), mice had significantly enlarged lateral ventricles compared to air-exposed controls (Air: Figure 2a; CAPS: Figure 2b), an observation confirmed by determination of lateral ventricle area using Neurolucida (MBF Bioscience, Williston, VT) that quantitated increases of 380% and 178% at PND14 and PND55, respectively (Figure 2c). On PND14 only, a single CAPS-treated female showed enlarged lateral

ventricle as indicated by increased variability in figure 2c for CAPS-females at that time point; however, this effect failed to reach statistical significance. Lateral ventricle size in mice (sex unspecified) have been reported to increase out to 90 days of age, with more rapid growth up to about 40 days of age (Mandell et al. 2010), although separate determinations by sex reveal that males show a decline in area between 1 and 3 mos of age (Mandell et al. 2010). Figure 2a shows representative images of lateral ventricles of PND14 male air- (Figure 2a) and CAPS-treated (Figure 2b) animals at similar bregma. CAPS did not affect aqueduct of Sylvius (Figure 2c). To further evaluate persistence, brains from identically exposed males obtained at PND270 were subjected to ventricular tracing and increased lateral ventricle size confirmed by a two-tailed t-test ($p=0.04$; Figure 2e,f).

To assess CAPS-induced glial activation as a potential mechanism of neurotoxicity, sections were immunostained for GFAP and IBA-1, markers for astrocytes and microglia, respectively. CAPS altered astrocyte state in a sex- and regionally-dependent manner (Figure 3 panels A-C)**.** Data is presented as percent time point- and sex-specific filtered air control values. In males, CAPS reduced GFAP immunostaining in the corpus callosum (main effect of CAPS: $F(1,15)=4.5986$, $p=0.048$), at both PND14 and PND55, while reductions in GFAP immunoreactivity in hippocampus occurred only at PND14 (time point x treatment interaction; $F(1,15)=5.200$, $p=0.0376$, $p<0.05$) (Figure 3, panels A and C). In contrast, females showed increases in GFAP in hippocampus (CAPS by time point: $F(1,16)=9.1589$,$p=0.008$), corpus callosum (CAPS by time point: $F(1,16=5.8066$, $p=0.0284$), and anterior commissure (CAPS by time point: $F(1,16)=12.76$, $p=0.018$) (Figure 3, panels B and C), that were restricted to PND14 relative to PND14 air-treated females (all p values <0.05), but not at PND55, indicating a

transient astrocytic response to CAPS present on PND14 that resolved by PND55. Data was also normalized to sex-specific PND14 filtered air control to allow for examination of trajectory of GFAP changes across the study period (see Supplemental Material, Figure S1).

CAPS altered IBA-1 immunostaining in the anterior commissure and hippocampus only in males (Figure 3, panels D and E). Data is presented as percent time point- and sex-specific filtered air control. Despite the larger increase at PND14, statistical analyses indicated that CAPS increased IBA-1 immunostaining (approximately 25%) in the anterior commissure at both time points (main effect of CAPS: $F(1,15)=5.75$, $p=0.03$), indicating persistent microglial response in the white matter. In contrast, CAPS increased IBA-1 immunoreactivity in hippocampus only at PND55 (CAPS by time point: $F(1,15)=4.8791$, $p=0.043$) relative to air-treated controls ($p<0.05$). Data was also normalized to sex-specific PND14 filtered air control to allow for examination of trajectory of IBA-1 changes across the study period (see Supplemental Material, Figure S2).

CAPS modified CNS neurotransmitter levels in a sex- and regionally-dependent manner. CAPS increased hippocampal glutamate ($F(1,26)=5.5383$, $p=0.0246$), midbrain DA TO ($F(1,26)=6.6590$, $p=0.0159$), and cortical DA TO ($F(1,18)=8.4456$, $p=0.0106$) in males at both time points (Table 1). CAPS increased cortical NE in males only at PND 55 (CAPS by time point: $F(1,26)=5.13$, $p=0.03$) (Table 2). No treatment-related differences in CNS neurotransmitters were observed in olfactory bulb, or hypothalamus nor in DA, DOPAC, HVA, 5-HT, or 5-HIAA in midbrain, striatum, cortex, or hippocampus (not shown).

In females, CAPS reduced hippocampal GABA ($F(1,28)=4.22$, $p=0.049$), but increased midbrain HVA ($F(1,29)=4.92$, $p= 0.035$) and DA ($F(1,29)=6.9$, $p=0.013$) and hippocampal serotonin ($F(1,29)=6.46$, $p=0.017$) at both time points (Table 2). Additionally, cortical NE was increased

only at PND55 (CAPS by time point: $F(1,29)=6.37$, $p= 0.017$), while hippocampal DA TO (CAPS by time point: $F(1,26)=4.90$, $p=0.036$) was increased, and midbrain DA TO reduced (CAPS by time point: $F(1,29)=9.52$, $p = 0.004$) only at PND14. No treatment-related differences in neurotransmitters in the olfactory bulb, cerebellum, or hypothalamus were observed (not shown).

In males, reductions in hippocampal IL-6 ($F(1,28)=4.69$, $p=0.039$), and in striatal IL-1β ($F(,1,28)=6.48$, $p= 0.017$) and TNFα ($F(1,28)=5.00$, $p=0.033$) were observed at both time points, with a similar trend in hippocampal IL-1β ($F(1,28)= 3.59$, $p= 0.069$) (Table 1). Hippocampal glutamate in CAPS-exposed males was positively correlated with hippocampal IL-1β ($r^2=0.233$, $p=0.039$) and IL-6 ($r^2=0.361$, $p=0.01$). In males, midbrain IL-1β (CAPS by time point: $F(1,28)=4.76$, $p=0.038$) and TNFα (CAPS by time point: $F(1,28)=5.448$, $p=0.027$) were reduced at PND14, but increased at PND55 (all p values <0.05) (Table 1). No treatment-related differences in central cytokines were observed in male olfactory bulb or cerebellum (not shown).

In females, CAPS reduced cortical IL-6 at PND55 (CAPS : $F(1,18)=5.78$, $p=0.027$) while increasing midbrain IL-6 (CAPS by time point: $F(1,28)=5.92$, $p=0.022$) at PND55 (Table 2). IL-6 in female cortex at PND14 was undetectable. Midbrain TNFα ($F(1,28)=7.05$, $p=0.013$) and IL-1β ($F(1,28)=6.65$, $p=0.016$) were increased at both time points (Table 2) In contrast, striatal IL-6 was increased at PND14, but not PND55 (CAPS by time point: $F(1,28)=8.61$, $p=0.007$, all post hoc p values <0.05) (Table 2). No treatment-related differences in cytokines were observed in olfactory bulb or cerebellum (not shown).

Discussion

Mice were exposed to human-relevant levels of UFP. As indicated in Figure 1, the average particle count was approximately 200,000 particles/cm^3. Ambient UFP counts near roadways in Los Angeles, California (Westerdahl et al. 2005) and Minneapolis, Minnesota (Kittelson 2004) have been reported as high has 200,000 and 400,000 particles/cm^3, respectively, with peak episodic counts reaching 2,000,000 particles/cm^3 in Minneapolis (Kittelson 2004).

CAPS induced a persistent dilation of the lateral ventricles, but not the aqueduct of Sylvius, preferentially in males. Lateral ventricle dilation is a predictor of poor neurodevelopmental outcome (Laskin et al. 2005; Tatli et al. 2012). It has been associated with multiple developmental CNS disorders, including autism and schizophrenia (Barttfeld et al. 2011; Bigler 1987; Fannon et al. 2000; Movsas et al. 2013; Sanfilipo et al. 2000; Schulz et al. 1983; Wright et al. 2000), idiopathic mental retardation, periventricular leukomalacia (Volpe 2005; Volpe 2003; Volpe 2001), fragile X syndrome and attention deficit disorder and, in the absence of other CNS abnormalities, to developmental delays (Gilmore et al. 2001; Gilmore et al. 2008). Its consequences can include progressive hydrocephalus, gray matter migration abnormalities, loss of parenchymal brain tissue, agenesis of the corpus callosum (CC) and delayed or abnormal maturation of white matter, i.e. reduced myelin basic protein (MBP) expression, diminished total axon volume, trisomies and microcephaly (Bigler 1987; Gilmore et al. 1998; Gilmore et al. 2001; Gilmore et al. 2008; Griffiths et al. 2010; Kuban et al. 1999; Kyriakopulou et al. 2013, Manfredi et al. 2010). Ventriculomegaly is associated with such deficits, persists after birth (Gilmore et al. 2001), and is more prevalent in males (Gilmore et al. 1998). Our observation of male-specificity of the lateral ventricle dilation is consistent with literature suggesting that males are more likely

to be diagnosed with a number of neurodevelopmental and neuropsychological disorders of childhood including autism, earlier on-set schizophrenia, attention deficit hyperactivity disorder, conduct disorder, and learning disabilities (CDC 2007; Kirkbride et al. 2012; Erskine et al. 2014; Boyle et al. 2011). While the mechanism(s) underlying the male-specificity of this effect are as yet undefined, they likely reflect sex differences in neurodevelopment such as the sex differences in microglial colonization of the brain seen already by PND4, at which time males show a more activated morphology (Schwarz et al. 2012), a possibility consistent with the observation that changes in IBA-1 were found only in males. Additionally, a single CAPS-exposed female appeared to show enlarged lateral ventricles at PND14 but considered across groups, these effects were not statistically significant, further suggesting male-specificity of the lateral ventricle dilation. However, future studies should address whether females may be rendered susceptible at higher particle concentrations or longer durations of exposure. Obstruction of the Aqueduct of Sylvius is a common mechanism of lateral ventricle dilation (James 1992) but was not seen here. Whether an earlier transient obstruction occurred cannot be ruled out, however. Future studies of a similar nature, should include utilization of repeated measures design in rodents exposed to CAPS. Use of magnetic resonance images (MRI) to track the trajectory of central ventricular system changes in the same animal across time would assist in illuminating the mechanism(s) by which such changes are induced by CAPS and may inform the sex-dependency of this effect. Global patterns of glial changes in the brain indicate that females mount a transient astroctyic response, but no microglial response to CAPS exposure, while males show both microglial and astrocytic dysfunction that persist into early adulthood.

Our previous work indicates significant disruption of adulthood neurotransmission in response to CAPS in mice that at least persists to almost 1 year of age (Allen et al 2014a,b). To determine the etiological role of such disruption in CAPS-induced neuropathology, regional levels of DA and its metabolites DOPAC and HVA, NE, 5-HT and its metabolite 5-HIAA, as well as glutamate, glutamine, and GABA were examined. Notably, the sustained increase in hippocampal glutamate may indicate the contribution of an excitotoxic mechanism of CAPS that persists until early adulthood. Additionally, increased dopamine metabolism, as evidenced in our animals by increased DA TO, has been associated with oxidative stress (Cohen 1983; Graham 1978; Hastings 1995; Schulz et al. 2000). Interestingly, loss of GABAergic neurons in the hippocampus, which is consistent with decreased hippocampal GABA observed in females here, has been implicated in both schizophrenia and bipolar disorder (Benes et al. 1998). Moreover, disrupted CNS neurotransmission is associated with both autism (Cook 1990) and schizophrenia (Grace 2012).

Early cytokine changes were also sex- and brain-region dependent (Tables 1 and 2). In the female midbrain, IL-6 was increased only at PND55, whereas TNFα and IL-1β were persistently increased across both time points. IL-6 in the female striatum was increased at PND14 only. A protracted profile of changes, as observed for female midbrain IL-6 may indicate adverse effects on ontogeny of microglial development that later results in a neuroinflammatory profile, while increases restricted to PND14 only, such as observed for female striatal IL-6, likely indicate a transient neuroinflammatory response.

Decreases, as opposed to increases, in male hippocampal IL-6 and striatal IL-1β/TNFα and in female cortical IL-6 were unanticipated, but perhaps suggest that microglia, a major source of

brain cytokines are dysfunctional or lost. Such cytokines have multiple important roles in the developing nervous system (Deverman and Patterson 2009), such that any alteration in brain cytokines during the early postnatal period would have deleterious effects on the CNS. Indeed, IL-1β, TNFα, and IL-6 have been implicated as having roles in synaptic plasticity in the hippocampus (Balschun et al. 2004; Goshen and Yirmiya 2009; Schneider et al. 1998), and can activate astrocytes that modulate synaptic plasticity. Furthermore, IL-1 receptor antagonist polymorphism was implicated in ADHD etiopathogenesis (Segman et al. 2002) and disrupted attention was observed in our male CAPS-exposed mice (Allen et al. 2013). Furthermore, hippocampal glu in CAPS-exposed males was positively correlated with hippocampal IL-1β/IL-6, likely indicating a mechanistic link between excitotoxicity and neuroinflammtory response. IL-1β has previously been proposed as a bridge between neuroinflammation and excitotoxicity (Fogal and Hewett 2008). This correlation was absent in air-exposed control males and in females regardless of exposure group.

Collectively these data show a dramatic susceptibility of male mice to environmentally relevant levels of early postnatal air pollution exposure, with effects that persist into adulthood and cause permanent neuropathology characterized by ventricular enlargement, a pathology not seen in females. Lateral ventricle dilation (ventriculomegaly), is a strong predictor of poor neurodevelopmental outcome in children and a pathological hallmark observed in both autism and schizophrenia. Thus, the current findings provide biological plausibility for the reported associations in epidemiological studies of air pollution with autism (Becerra et al. 2013; Volk et al. 2011; Volk et al. 2013), schizophrenia (Pedersen et al. 2004), and ADHD (Siddique et al.

2011) Moreover, the heightened sensitivity of males to CAPS effects parallels the greater prevalence of these disorders in males.

Although CAPS-induced ventricular enlargement is not observed in females, CAPS-exposed females exhibit biochemical and neurochemical alterations that are nevertheless significant and represent protracted neurotoxicity in response to early postnatal CAPS exposure. Males and females show significantly altered neurochemical changes in multiple brain regions, including areas that comprise the mesocorticolimbic dopamine tracts, regions critical to cognition and attention. CAPS-exposed males have increased levels of major excitatory neurotransmitter glutamate in hippocampus, a sign of excitotoxicity in that region. CAPS-exposed females show a decrease in hippocampal GABA, the major inhibitory neurotransmitter of the CNS. GABA alterations in the hippocampus have been implicated in both schizophrenia and bipolar disorder (Benes and Berretta 2001). The functional/behavioral significance of these changes remains to be fully determined; however impairment in behaviors involving the hippocampus, such as learning and memory, would be predicted. These changes in neurotransmitters along the mesocorticolimbic pathway may underlie the increased preference for immediate reward observed in CAPS-exposed males that we previously reported (Allen et al. 2013). In interpreting these findings, inherent differences between murine and human brain development must also be considered. The early postnatal period in both humans and rodents is marked by substantial brain development; however, the exact nature of the development is different. As a rough estimate, rat brain development at PND7 has been equated to approximate brain development at birth in the human (Clancy et al. 2007), thus our exposure paradigm that occurred from PND 4-7 and 10-13 in mice, in terms of neurodevelopment, probably best approximates what would be the perinatal

period in the human encompassing the timeframe shortly before and after birth. Taken together, these data suggest that exposure to CAPS in the early postnatal period, at human- and environmentally-relevant levels, may represent a far greater public health concern than has previously been recognized as a risk factor contributing to intractable neurodevelopmental disorders such as autism and schizophrenia.

References

Allen JL, Conrad K, Oberdorster G, Johnston CJ, Sleezer B, Cory-Slechta DA. 2013. Developmental exposure to concentrated ambient particles and preference for immediate reward in mice. Environ Health Perspect 121:32-38.

Allen JL, Liu X, Weston D, Prince L, Oberdorster G, Finkelstein JN et al. 2014a. Developmental exposure to concentrated ambient ultrafine particulate matter air pollution in mice results in persistent and sex-dependent behavioral neurotoxicity and glial activation. Toxicol Sci [epub ahead of print].

Allen JL, Liu X, Weston D, Conrad K, Oberdorster G, Cory-Slechta DA. 2014b. Consequences of developmental exposure to concentrated ambient ultrafine particle air pollution combined with the adult paraquat and maneb model of the Parkinson's disease phenotype in male mice. Neurotoxicology 41:80-88.

Balschun D, Wetzel W, Del Rey A, Pitossi F, Schneider H, Zuschratter W, et al. 2004. Interleukin-6: A cytokine to forget. FASEB J 18:1788-1790.

Barttfeld P, Wicker B, Cukier S, Navarta S, Lew S, Sigman M. 2011. A big-world network in ASD: dynamical connectivity analysis reflects a deficit in long-range connections and an excess of short-range connections. Neuropsychologia 49(2):254-263.

Becerra TA, Wilhelm M, Olsen J, Cockburn M, Ritz B. 2013. Ambient air pollution and autism in los angeles county, california. Environ Health Perspect 121:380-386.

Benes FM, Kwok EW, Vincent SL, Todtenkopf MS. 1998. A reduction of nonpyramidal cells in sector ca2 of schizophrenics and manic depressives. Biol Psychiatry 44:88-97.

Benes BM, Berretta S. 2001. GABAergic Interneurons: Implications for Understanding Schizophrenia and Bipolar Disorder. Neuropsychopharmacology 25:1-27.

Bigler ED. 1987. The clinical significance of cortical atrophy and ventricular enlargement in schizophrenia. Arch Clin Neuropsychol 2(4):385-392.

Block ML, Calderon-Garciduenas L. 2009. Air pollution: Mechanisms of neuroinflammation and CNS disease. Trends Neurosci 32:506-516.

Boyle CA, Boulet S, Schieve LA, Cohen RA, Blumberg SJ, Yeargin-Allsopp M. 2011. Trends in the prevalence of developmental disabilities in US children, 1997-2008. Pediatrics 6:1034-1042.

Cao RY, St Amand T, Li X, Yoon SH, Wang CP, Song H, et al. 2012. Prostaglandin receptor EP4 in abdominal aortic aneurysms. Am J Pathol 181(1): 313-321.

CDC. 2007. Prevalence of autism spectrum disorders --autism and developmental disabilities monitoring network. MMWR Surveill Summ 68:1-11.

Clancy B, Finlay BL, Darlington RB, Anand KJ. 2007. Extrapolating brain development from experimental species to humans. Neurotoxicology 28:931-937.

Cohen G. 1983. The pathobiology of Parkinson's disease: biochemical aspects of dopamine neuron senescence. J Neural Transm Suppl 19:89-103.

Cook EH. 1990. Autism: Review of neurochemical investigation. Synapse 6:292-308.

Cory-Slechta DA, Virgolini MB, Thiruchelvam M. Weston DD, Bauter MR. 2004. Maternal stress modulates the effects of developmental lead exposure. Environ Health Perspect 112: 717-730.

Cory-Slechta DA, Weston D, Liu S, Allen JL. 2013. Brain hemispheric differences in the neurochemical effects of lead, prenatal stress, and the combination and th eir amelioration by behavioral experience. Toxicol Sci 132(2):419-430.

Deverman BE, Patterson PH. 2009. Cytokines and CNS development. Neuron 64:61-78.

Erskine HE, Ferrari AJ, Polanczyk GV, Moffitt TE, Murray CJ, Vos T, et al. 2014. The global burden of conduct disorder and attention-deficit/hyperactivity disorder in 2010. J Child Psychol Psychiatry 4:328-336.

Fannon D, Tennakoon L, Sumich A, O'Ceallaigh S, Doku V, Chitnis X, et al. 2000. Third ventricle enlargement and developmental delay in first-episode psychosis: preliminary findings. Br J Psychiatry 177:354-359.

Fogal B, Hewett SJ. 2008. Interleukin-1beta: A bridge between inflammation and excitotoxicity? J Neurochem 106:1-23.

Gilmore JH, van Tol J, Kliewer MA, Silva SG, Cohen SB, Hertzberg BS, et al. 1998. Mild ventriculomegaly detected in utero with ultrasound: Clinical associations and implications for schizophrenia. Schizophr Res 33:133-140.

Gilmore JH, van Tol JJ, Lewis Streicher H, Williamson K, Cohen SB, Greenwood RS, et al. 2001. Outcome in children with fetal mild ventriculomegaly: a case series. Schizophr Res 48(2-3):219-226.

Gilmore JH, Smith LC, Wolfe HM, Hertzberg BS, Smith JK, Chescheir NC, et al. 2008. Prenatal mild ventriculomegaly predicts abnormal development of the neonatal brain. Biol psychiatry 64(12): 1069-1076.

Goshen I, Yirmiya R. 2009. Interleukin-1 (il-1): A central regulator of stress responses. Front Neuroendocrinol 30:30-45.

Grace AA. 2012. Dopamine system dysregulation by the hippocampus: Implications for the pathophysiology and treatment of schizophrenia. Neuropharmacology 62:1342-1348.

Graham DG. 1978. Oxidative pathways for catecholamines in genesis of neuromelanin and cytotoxic quinones. Mol Pharmacol 14(4):633-643.

Griffiths PD, Reeves MJ, Morris JE, Mason G, Russell SA, Paley MN, et al. 2010. A prospective study of fetuses with isolated ventriculomegaly investigated by antenatal sonography and in utero MR imaging. AJNR AM J Neuroradiol 31(1):106-111.

Hastings TG. 1995. Enzymatic oxidation of dopmaine: The role of prostaglandin H synthase. J Neurochem 64(2): 919-924.

James HE. 1992. Hydrocephalus in infancy and childhood. American family physician 45:733-742.

Kirkbride JB, Errazuriz A, Croudace TJ, Morgan C, Jackson D, Boydell J, et al. 2012. Incidence of schizophrenia and other psychoses in england, 1950-2009: A systematic review and meta-analyses. PLoS One 7(3):e31660.

Kittelson DB. 2004. Nanoparticle emissions on minnesota highways. Atmos Environ 38:9-19.

Laskin MD, Kingdom J, Toi A, Chitayat D, Ohlsson A. 2005. Perinatal and neurodevelopmental outcome with isolated fetal ventriculomegaly: A systematic review. J Matern Fetal Neonatal Med 18(5):289-298.

Kuban K, Sanocka U, Leviton A, Allred EN, Pagano M, Dammann O, et al. 1999. White matter disorders of prematurity: association with intraventricular hemorrhage and ventriculomegaly. The Developmental Epidemiology Network. J Pediatr 134(5):539-546.

Kyriakopoulou V, Vatansever D, Elkommos S, Dawson S, McGuinness A, Allsop J, et al. 2013. Cortical Overgrowth in Fetuses With Isolated Ventriculomegaly. Cerebral Cortex; doi: 10.1093/cercor/bht062 [Online 18 March2013]

Lim YH, Kim H, Kim JH, Bae S, Park HY, Hong YC. 2012. Air pollution and symptoms of depression in elderly adults. Environ Health Perspect 120:1023-1028.

Lisabeth LD, Escobar JD, Dvonch JT, Sanchez BN, Majersik JJ, Brown DL, et al. 2008. Ambient air pollution and risk for ischemic stroke and transient ischemic attack. Ann Neurol 64:53-59.

Mandell JG, Neuberger T, Drapaca CS, Webb AG, Schiff SJ. 2010. The dynamics of brain and cerebrospinal fluid growth in normal versus hydrocephalic mice. J Neurosurg Pediatr 6:1-10.

Manfredi R, Tognolini A, Bruno C, Raffaelli R, Franchi M, Pozzi Mucelli R. 2010. Agenesis of the corpus callosum in fetuses with mild ventriculomegaly: role of MR imaging. Radiol Med 115(2):301-312.

Movsas TZ, Pinto-Martin JA, Whitaker AH, Feldman JF, Lorenz JM, Korzeniewski SJ, et al. 2013. Autism Spectrum Disorder Is Associated with Ventricular Enlargement in a Low Birth Weight Population. J Pediatr 163(1):73-78.

Oberdorster G. 2000. Toxicology of ultrafine particles: In vivo studies. Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences 358(1775):2719-2740.

Pedersen CB, Raaschou-Nielsen O, Hertel O, Mortensen PB. 2004. Air pollution from traffic and schizophrenia risk. Schizophr Res 66:83-85.

Power MC, Weisskopf MG, Alexeeff SE, Coull BA, Spiro A, 3rd, Schwartz J. 2011. Traffic-related air pollution and cognitive function in a cohort of older men. Environ Health Perspect 119:682-687.

Sanfilipo M, Lafargue T, Arena L, Rusinek H, Kushner K, Lautin A, et al. 2000. Fine volumetric analysis of the cerebral ventricular system in schizophrenia: further evidence for multifocal mild to moderate enlargement. Schizophr Bull 26(1):201-216.

Schneider H, Pitossi F, Balschun D, Wagner A, del Rey A, Besedovsky HO. 1998. A neuromodulatory role of interleukin-1beta in the hippocampus. Proc Natl Acad Sci U S A 95:7778-7783.

Schulz JB, Lindenau, Seyfried J, Dichgans J. 2000. Glutathione, oxdiative stress and neurodegeneration. Eur J Biochem 267: 4904-4911.

Schulz SC, Koller MM, Kishore PR, Hamer RM, Gehl JJ, Friedel RO. 1983. Ventricular enlargement in teenage patients with schizophrenia spectrum disorder. Am J Psychiatry 140(12):1592-1595.

Schwarz JM, Sholar PW, Bilbo SD. 2012. Sex differences in microglial colonization of the developing rat brain. J Neurochem 120:948-963.

Segman RH, Meltzer A, Gross-Tsur V, Kosov A, Frisch A, Inbar E, et al. 2002. Preferential transmission of interleukin-1 receptor antagonist alleles in attention deficit hyperactivity disorder. Mol Psychiatry 7:72-74.

Siddique S, Banerjee M, Ray MR, Lahiri T. 2011. Attention-deficit hyperactivity disorder in children chronically exposed to high level of vehicular pollution. Eur J Pediatr 7:923-929.

Tatli B, Ozer I, Ekici B, Kalelioglu I, Has R, Eraslan E, et al. 2012. Neurodevelopmental outcome of 31 patients with borderline fetal ventriculomegaly. Clin Neurol Neurosurg 114:969-971.

Virgolini MB, Rossi-George A, Lisek R, Weston DD, Thiruchelvam M, Cory-Slechta DA. 2008. CNS effects of developmental Pb exposure are enhanced by combined maternal and offspring stress. Neurotoxicology 29: 812-827.

Volk HE, Hertz-Picciotto I, Delwiche L, Lurmann F, McConnell R. 2011. Residential proximity to freeways and autism in the charge study. Environ Health Perspect 119:873-877.

Volk HE, Lurmann F, Penfold B, Hertz-Picciotto I, McConnell R. 2013. Traffic-related air pollution, particulate matter, and autism. JAMA psychiatry 70:71-77.

Volpe JJ. 2001. Neurobiology of periventricular leukomalacia in the premature infant. Pediatr Res50(5): 553-562.

Volpe JJ. 2003. Cerebral white matter injury of the premature infant-more common than you think. Pediatrics 112(1 Pt 1): 176-180.

Volpe JJ. 2005. Encephalopathy of prematurity includes neuronal abnormalities. Pediatrics 116(1): 221-225.

Wellenius GA, Burger MR, Coull BA, Schwartz J, Suh HH, Koutrakis P, et al. 2012. Ambient air pollution and the risk of acute ischemic stroke. Arch Intern Med 172:229-234.

Westerdahl D, Fruin S, Sax T, Fine PM, Sioutas C. 2005. Mobile platform measurments of ultrafine particles and associated pollutant concentrations on freeways and residential stresses in Los Angeles. Atmos Environ 39:3597-3610.

Weuve J, Puett RC, Schwartz J, Yanosky JD, Laden F, Grodstein F. 2012. Exposure to particulate air pollution and cognitive decline in older women. Arch Intern Med 172:219-227.

Wright IC, Rabe-Hesketh S, Woodruff PW, David AS, Murray RM, Bullmore ET. 2000. Meta-analysis of regional brain volumes in schizophrenia. Am J of Psychiatry 157(1): 16-25.

Table 1. Neurochemical disruption and cytokine changes in hippocampus, cortex, midbrain, and striatum of CAPS exposed males.

Exposure	DA TO	NE	IL-1β	TNFα	IL-6	Glu
Hippocampus						
PND14						
Air	NA	NA	3.36 ± 0.77	NA	0.58 ± 0.15	41.57 ± 1.46
CAPS	NA	NA	2.39 ± 0.52	NA	0.35 ± 0.09	52.47 ± 4.80
PND55						
Air	NA	NA	2.76 ± 0.16	NA	0.61 ± 0.05	52.27 ± 2.92
CAPS	NA	NA	2.09 ± 0.24	NA	0.45 ± 0.07	54.85 ± 3.03
Overall Effects	NA	NA	TX	NA	TX	TX
Cortex						
PND14						
Air	22.35 ± 7.11	3.29 ± 0.17	0.97 ± 0.13	NA	NA	NA
CAPS	47.47 ± 21.16	3.29 ± 0.09	0.74 ± 0.06	NA	NA	NA
PND55						
Air	3.43 ± 0.93	7.61 ± 0.31	2.71 ± 0.48	NA	NA	NA
CAPS	6.45 ± 1.57	8.82 ± 0.31*	1.70 ± 0.20	NA	NA	NA
Overall Effects	TP, TX, TP x TX	TP, TX, TP x TX	TP, TX	NA	NA	NA
Midbrain						
PND14						
Air	4.26 ± 0.28	NA	20.33 ± 4.50	3.47 ± 0.76	NA	NA
CAPS	5.75 ± 0.62	NA	10.23 ± 0.911*	1.80 ± 0.15*	NA	NA
PND55						
Air	1.01 ± 0.13	NA	19.99 ± 1.86	3.38 ± 0.29	NA	NA
CAPS	1.23 ± 0.20	NA	27.85 ± 6.710*	4.90 ± 1.12*	NA	NA
Overall Effects	TP, TX	NA	TP, TP x TX	TP, TX x TX	NA	NA
Striatum						
PND14						
Air	NA	NA	2.11 ± 0.37	0.93 ± 0.23	NA	NA
CAPS	NA	NA	1.57 ± 0.24	0.52 ± 0.11	NA	NA
PND55						
Air	NA	NA	1.39 ± 0.30	0.20 ± 0.04	NA	NA
CAPS	NA	NA	0.53 ± 0.11	0.10 ± 0.02	NA	NA
Overall Effects	NA	NA	TP, TX	TX	NA	NA

Table 2. Neurochemical disruption and cytokines changes in hippocampus, cortex, midbrain, and striatum of CAPS exposed females.

Exposure	DA TO	NE	DA	HVA	5-HT	IL-1β	TNFα	IL-6	GABA
Hippocampus									
PND14									
Air	72.26 ± 15.97	NA	NA	NA	8.65 ± 0.49	NA	NA	NA	3.68 ± 0.10
CAPS	99.94 ± 8.45*	NA	NA	NA	9.21 ± 0.53	NA	NA	NA	2.81 ± 0.35
PND55									
Air	18.32 ± 5.68	NA	NA	NA	22.46 ± 1.34	NA	NA	NA	4.87 ± 0.45
CAPS	13.64 ± 2.44	NA	NA	NA	28.30 ± 1.33*	NA	NA	NA	4.23 ± 0.32
Overall Effects	TP, TP x TX	NA	NA	NA	TP, TX, TP X TX	NA	NA	NA	TP, TX
Cortex									
PND14									
Air	NA	3.46 ± 0.24	NA	NA	NA	NA	NA	n.d.	NA
CAPS	NA	3.54 ± 0.13	NA	NA	NA	NA	NA	n.d.	NA
PND55									
Air	NA	8.27 ± 0.23	NA	NA	NA	NA	NA	0.36 ± 0.08	NA
CAPS	NA	7.25 ± 0.201*	NA	NA	NA	NA	NA	0.18 ± 0.028*	NA
Overall Effects	NA	TP, TX, TP x TX	NA	NA	NA	NA	NA	NA	NA
Midbrain									
PND14									
Air	5.85 ± 0.69	NA	1.83 ± 0.17	5.40 ± 0.33	NA	11.50 ± 2.07	2.13 ± 0.32	1.55 ± 0.31	NA
CAPS	3.83 ± 0.40*	NA	2.81 ± 0.25	6.51 ± 0.39	NA	13.27 ± 1.89	2.39 ± 0.24	1.56 ± 0.23	NA
PND55									
Air	1.34 ± 0.11	NA	2.19 ± 0.18	2.30 ± 0.16	NA	14.95 ± 1.45	2.72 ± 0.24	2.34 ± 0.26	NA
CAPS	1.29 ± 0.08	NA	2.37 ± 0.21	2.28 ± 0.13	NA	27.72 ± 3.12	4.77 ± 0.49	4.84 ± 0.588*	NA
Overall Effects	TP, TX, TP x TX	NA	TX	TP, TX	NA	TX	TP, TX	TP, TX, TP x TX	NA
Striatum									
PND14									
Air	NA	NA	NA	NA	NA	NA	0.19 ± 0.02	0.40 ± 0.14	NA
CAPS	NA	NA	NA	NA	NA	NA	0.13 ± 0.019*	0.15 ± 0.034*	NA
PND55									
Air	NA	NA	NA	NA	NA	NA	0.11 ± 0.02	0.05 ± 0.01	NA
CAPS	NA	NA	NA	NA	NA	NA	0.12 ± 0.02	0.07 ± 0.01	NA
Overall Effects	NA	NA	NA	NA	NA	NA	TP, TX, TP x TX	TP, TX, TP x TX	NA

Dopamine turnover (DA TO), Norepinephrine NE), Dopamine (DA), Homovanillic Acid (HVA), Serotonin (5-HT), Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα), Interleukin-6 (IL-6), and γ-aminobutyric acid (GABA). Data reported as group mean ± SE. Monamines (DA TO, NE, DA, HVA, and 5-HT) reported in ng/mg protein, amino acids (GABA) reported in μg/mg protein, Cytokines (IL-1β, TNFα, IL-6) reported in pg/mg protein). (n=8-12 animals/treatment/time point). * indicates statistical difference ($p<0.05$) from time point-specific air control. TP and/or TX indicate statistical main effect of time point and/or treatment, respectively. TP x TX indicates statistical interaction.

Figure legends

Figure 1. Mean particle counts (left axis) and particle mass concentration (right axis) ± SD for each day of exposure. Mean diameter (inset) ± SD for each day of exposure.

Figure 2. Images of male lateral ventricle in Air-exposed (A) and CAPS-exposed (B) male mice from at PND14 (scale bar is 10 μm) or approximately PND270 (scale bar is 100μm) after exposure (D and E, respectively). Quantification of lateral ventricle on PND14, 55, and 270 (C/F) and Aqueduct of Sylvius area on PND14 and 55 is below (C). Data reported as group mean area ± SE. (n=5 animals/sex/treatment/time point). TX indicates main effect of CAPS treatment*indicates results of two-tailed t-test, $p<0.05$.

Figure 3. Representative Images of GFAP Immunoreactivity in males (A) and females (B) in the corpus callosum and hippocampus of Air- and CAPS-exposed mice at PND14 with relative quantification in those regions and the dentate gyrus, cortex, midbrain, striatum, and anterior commissure immediately adjacent (C). Images of IBA-1 immunoreactivity in anterior commissure at PND14 and hippocampus of male mice at PND55 (D) with relative quantification immediately below (E). Data reported as percent sex-specific control by time point ± SE. (n=5 animals/sex/treatment /time point). Bar=50 μm. TX indicates main effect of CAPS treatment and TP x TX indicates statistical interaction between CAPS treatment and time point. * indicates statistically different ($p<0.05$, two-tailed) from time point- and sex-specific control.

Figure 1.

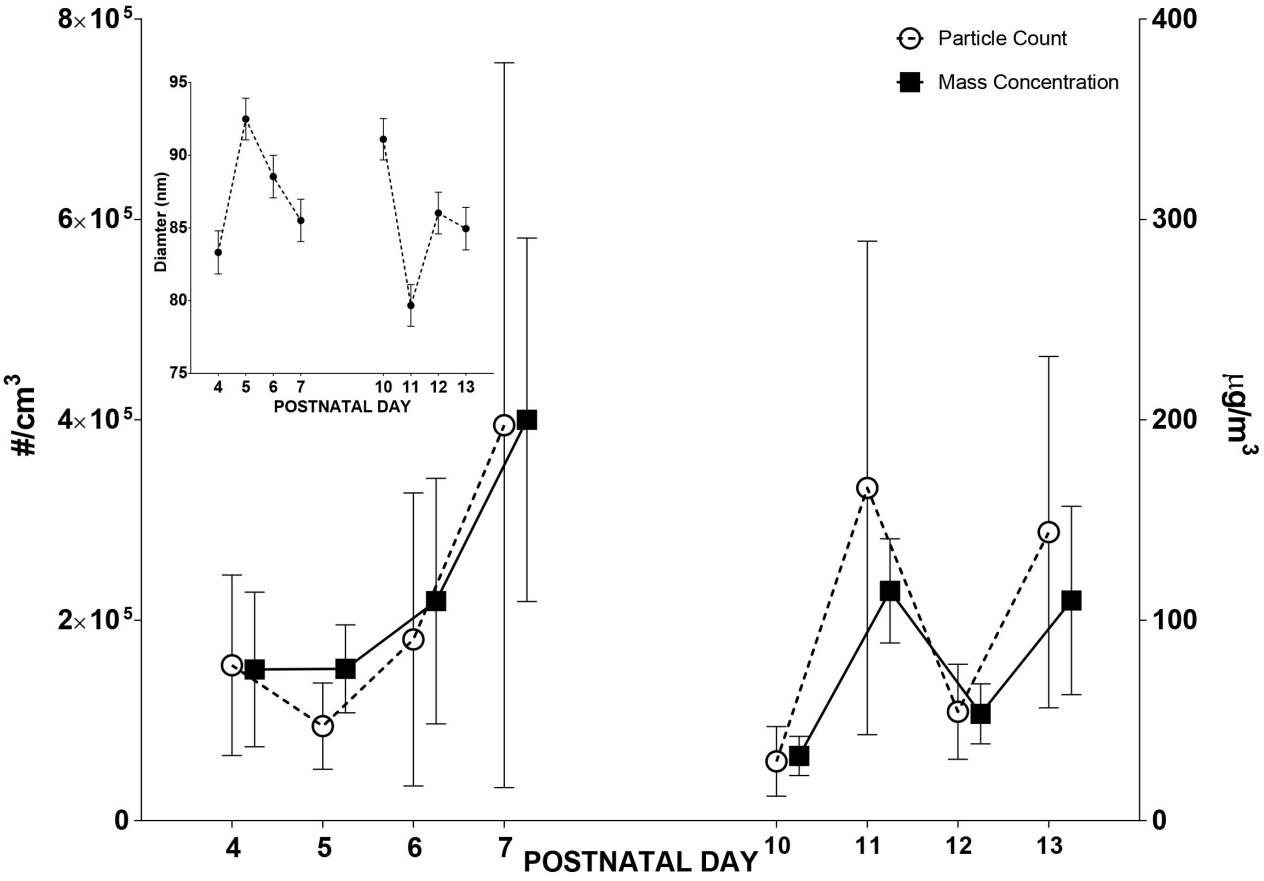
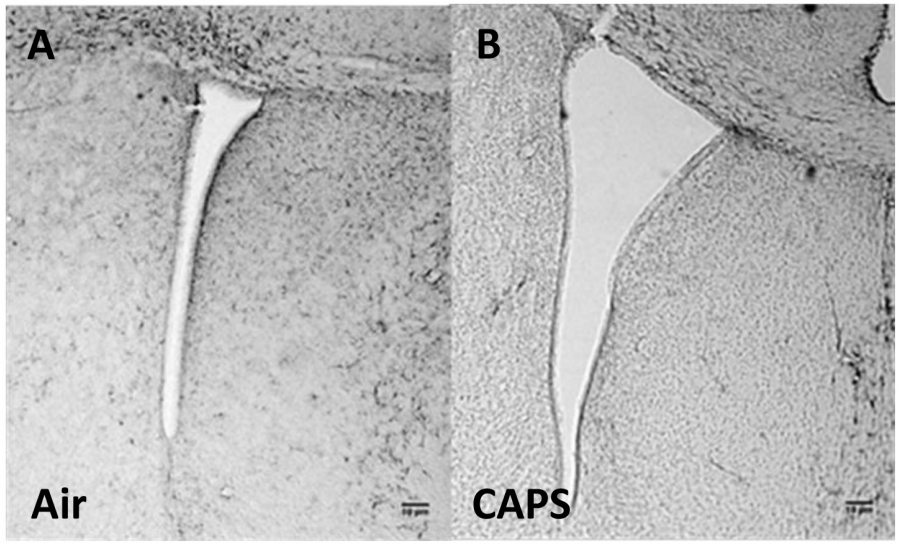
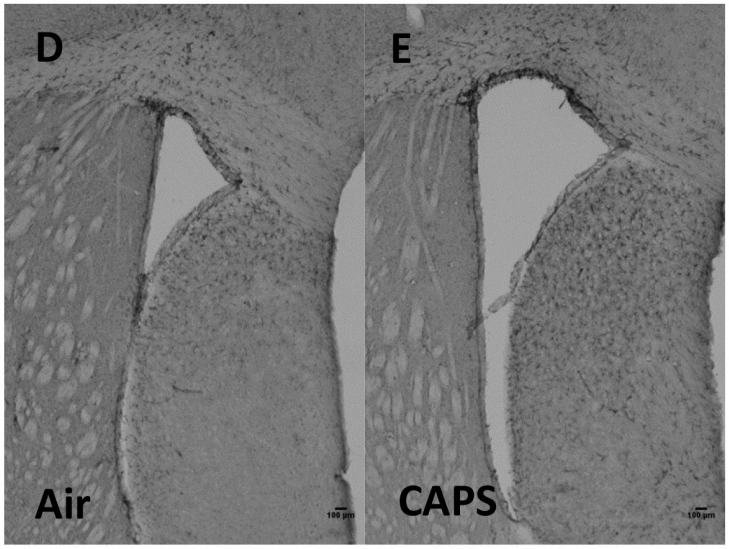
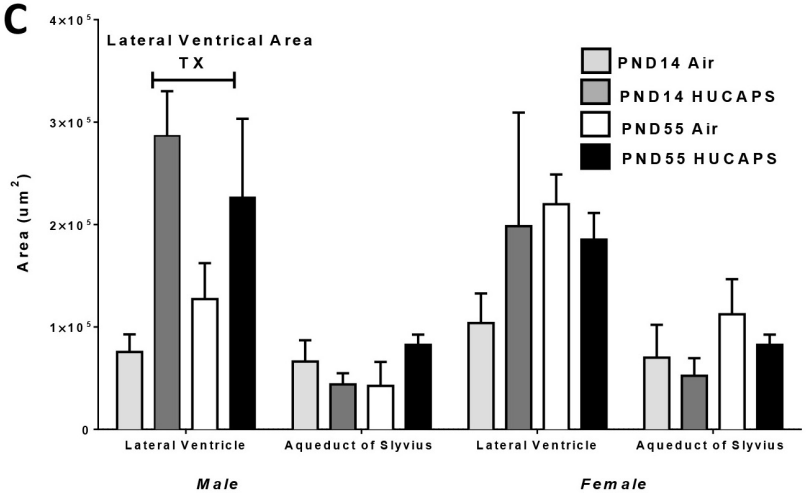

Particle Count
Mass Concentration
#/cm3
μg/m3
POSTNATAL DAY
Diamter (nm)
POSTNATAL DAY

Figure 2.


A
B
Air
CAPS


D
E
Air
CAPS


C
Lateral Ventrical Area
TX
PND14 Air
PND14 HUCAPS
PND55 Air
PND55 HUCAPS
Area (um2)
4x10 5
3x10 5
2x10 5
1x10 5
0
Lateral Ventricle
Aqueduct of Slyvius
Lateral Ventricle
Aqueduct of Slyvius
Male
Female

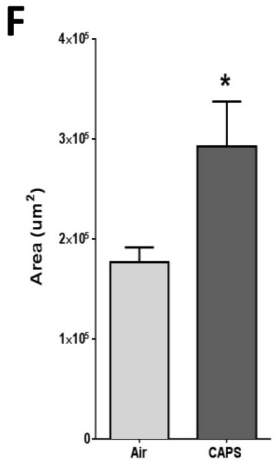
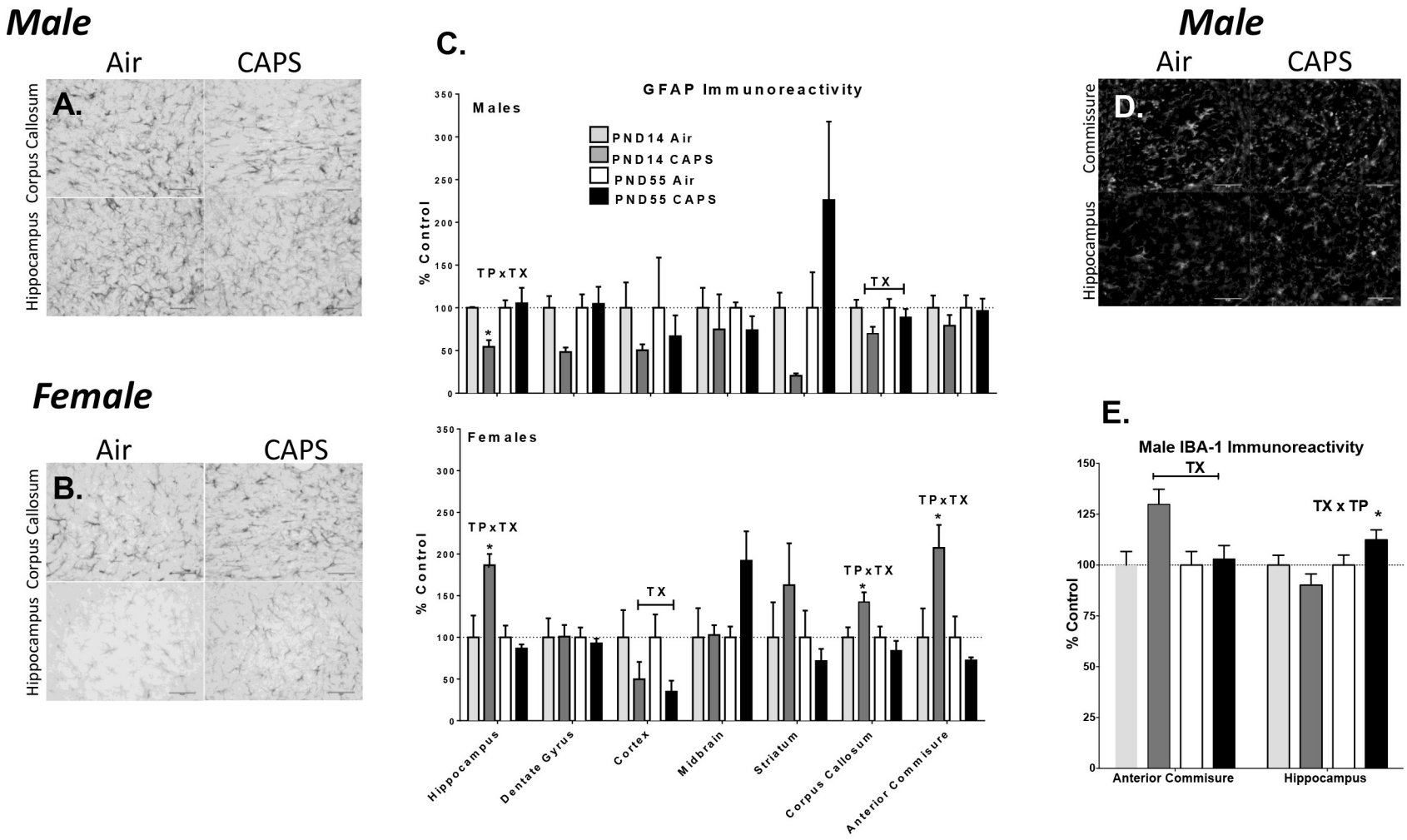

F
4x10^5
3x10^5
2x10^5
1x10^5
0
Area (um2)
*
Air
CAPS

Figure 3.


Male
Air
CAPS
A.
Hippocampus Corpus Callosum
Female
B.
C.
GFAP Immunoreactivity
Males
PND14 Air
PND14 CAPS
PND55 Air
PND55 CAPS
% Control
TPxTX
TX
Females
Hippocampus
Dentate Gyrus
Cortex
Midbrain
Striatum
Corpus Callosum
Anterior Commisure
D.
Commissure
Hippocampus
E.
Male IBA-1 Immunoreactivity
TX x TP